GENE FUSION ANALYSIS OF POSITIVE CHARGE-INDUCED SEGMENT RE-ORIENTATION IN THE TETRACYCLINE RESISTANCE PROTEIN

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ABSTRACT

Title of Thesis: GENE FUSION ANALYSIS OF POSITIVE CHARGE-INDUCED SEGMENT RE-ORIENTATION IN THE TETRACYCLINE RESISTANCE PROTEIN

Farrell McAfee, Master of Science, 1993

Thesis directed by: Kurt Miller, PhD. Department of Biochemistry

The pBR322 tetracycline resistance protein (Tet) was used as a model system for analysis of control of transmembrane segment orientation in membrane proteins. Two types of Tet fusions were used to test the effects of positively-charged amino acids and tertiary interactions within the protein on segment orientation. The influence of tertiary interactions was examined using fusions where maltose binding protein (MBP) was linked to the N-terminus of the full-length Tet protein. Local charge control was examined using truncated Tet-alkaline phosphatase (PhoA) fusions where C-terminal segments were deleted from Tet. Three lysine residues were introduced into the third periplasmic domain of Tet to reverse the positive charge balance across its fifth transmembrane segment. The introduction of lysine residues into the intact Tet domain of a MBP-Tet fusion abolished tetracycline resistance of the fusion and made the fusion unstable to proteolysis both *in vivo* and *in vitro*. While these data suggest the structure of the Tet domain was altered, rearrangement of segments could not be confirmed directly by proteolysis due to the instability of the protein. A comparison of the alkaline phosphatase activities of wild-type and mutant Tet PhoA fusions indicated that positively-charged residues are sufficient to cause segment inversion in the absence of part of the Tet domain.

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by

Farrell McAfee

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DEDICATION

To my family:

Karen, Nathan, Austin, Devin, and Brenna

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I. INTRODUCTION

All eukaryotic and many prokaryotic cells are divided into multiple membrane or membrane-bounded compartments, each containing a unique set of resident proteins. *Escherichia coli* has four distinct cell compartments: cytoplasm, inner (plasma) membrane, periplasm and outer membrane. Cellular protein synthesis takes place in the cytoplasm on free or membrane-associated ribosomes. The process of directing a protein to a particular membrane compartment and the subsequent insertion of the protein into it is known as targeting. A transmembrane protein not only must be targeted to the correct cellular compartment but also must contain information within its sequence for determining its membrane topology.

It has been suggested that each hydrophobic transmembrane segment of a membrane protein may behave as an independent entity that contains topogenic information specifying its orientation in the plane of the membrane (Bibi, *et. al.*, 1991). Emerging evidence indicates that positively-charged amino acid residues, particularly arginyl and lysyl residues, are determinants of membrane protein topology (von Heijne, 1986, Dalbey, 1990, and Hartmann, *et. al.*, 1987). According to the "positive inside rule" (von Heijne, 1988), hydrophobic transmembrane segments orient themselves in the membrane with their most positive ends toward the cytoplasm. Comparisons of the charge distribution in sequences flanking the most amino-terminal transmembrane segments of 91 eukaryotic proteins with uncleaved signals (Hartman, *et. al.*, 1989) demonstrated a correlation between transmembrane segment orientation and charge difference from carboxy-terminal and amino-terminal flanking residues. A negative charge difference between the 15 carboxy-terminal and

15 amino-terminal flanking residues [Δ(C - N)<0] correlated with the amino terminus of the transmembrane segment facing the cytoplasm (N_{cyt}/C_{exo} orientation). A positive difference [Δ(C - N)$\geq$0] correlated with an N_{exo}/C_{cyt} orientation. The reliability of predicting transmembrane segment orientation based on flanking charged residues was also supported by analyzing a series of mutants of the asialoglycoprotein (ASGP) receptor subunit H1 protein, a single transmembrane-spanning (bitopic) protein (Beltzer, *et. al.*, 1991). However, there may be exceptions to the "positive inside rule". Other features of protein structure can affect the insertion process, *e.g.*, folded protein domains adjacent to hydrophobic segments may not be competent for translocation even in the presence of the appropriate charge signals (Beltzer, *et. al.*, 1991).

Escherichia coli (E. coli) is an excellent model organism for the study of translocation mechanisms. Translocation in *E. coli* is thought to be catalyzed by at least two components of the secretion machinery; SecB and translocase. SecB, a chaperonin, prevents premature folding in some secretory proteins and may participate in delivery of preproteins to their target site on the membrane surface (Collier, *et. al.*, 1988). Translocase consists of at least two proteins: the SecA protein, an ATPase primary receptor for the SecB/preprotein complex that is peripheral to the translocase, and the membrane-embedded SecY/E protein which stabilizes and activates SecA and participates in the binding of the preprotein to the membrane (Cunningham, *et. al*, 1989). Initiation of translocation is driven by the binding of ATP to SecA and involves the relocation of the amino-terminus of preproteins from a SecA-bound to a membrane-inserted state (Schiebel, *et. al.*,

1991). Preproteins subsequently are threaded through the membrane by many cycles of SecA binding and ATP hydrolysis which pushes the protein through the membrane in discreet steps. The translocation steps may also require the membrane potential. Translocated proteins targeted to the periplasm or outer membrane are processed by endoproteolytic cleavage from leader peptidase, whereas most inner membrane proteins lack cleavable signal peptides. It is not known how the secretion machinery may recognize the sign of charged residues flanking transmembrane segments to fix their membrane orientation.

In cases where short peptide segments are translocated across a membrane, *e.g.*, the M13 phage procoat protein, and the bee toxin preprotein, prepromelittin, translocation has been shown to be independent of any secretion machinery (Wolfe *et. al.*, 1985, and Cobet, *et. al.*, 1989). The M13 coat protein precursor was observed to insert spontaneously in the correct orientation into protein-free liposomes (Geller and Wickner, 1985), demonstrating the presence of topogenic information in the protein's sequence. The Sec-dependent insertion of membrane proteins cannot simply be a question of their size, as the 40-kDa MalF protein requires neither SecA nor SecY for its membrane insertion (McGovern and Beckwith, 1991).

The pBR322 tetracycline resistance protein (Tet) is the model selected for studying the control of transmembrane segment insertion in this research. Hydrophobicity profiles of the 43-kDa cytoplasmic membrane, metal-tetracycline/H^+ antiporter (Yamaguchi, *et. al.*, 1990) predict 12 potential transmembrane segments with 6 periplasmic loops and 5 cytoplasmic loops (Figure 1). The two-dimensional, 12 membrane-spanning segment structure is supported by proteolysis of the related

Tn10 Tet protein in inverted membrane vesicles (IMVs) (Eckert and Beck, 1989) and alkaline phosphatase fusions of pBR322 (Allard and Bertrand, 1992).

The tertiary structures of only a few membrane proteins, *e.g.*, the bacterial photosynthetic reaction center complex (Diesenhofer, *et. al.*, 1985) and bacteriorhodopsin (Huang, *et. al.*, 1982) have been determined to high resolution because growing crystals suitable for diffraction analysis is difficult. Gene fusion, proteolysis and mutational analysis are alternative methods used in this research for monitoring the changes of topology in Tet. The classical proteolysis mapping approach assumes that domains protruding from the membrane are accessible to proteases, whereas the regions embedded in the lipid bilayer are shielded. Proteolytic digestions of membrane proteins can be performed from the outside, using spheroplasts, or inside, using IMV's. Fragment size analysis from proteolysis yields information on the protein topology. Another method of gathering information on topology is the technique of fusing the alkaline phosphatase reporter to selected sites. Alkaline phosphatase (PhoA) fusions have been used to examine signals involved in cell envelope and secreted proteins (Manoil, *et. al.*, 1990). An important property of the PhoA fusion is that the enzyme domain is active only when it is exported across the cytoplasmic membrane into the periplasmic space. Because of this property, the alkaline phosphatase can be used as a sensor to locate protein export signals.

The prototype protein selected for the proteolysis study is the N-terminal recombinant fusion protein between the maltose binding protein (MBP) and pBR322 Tet (Figure 2). The signal peptide sequence is deleted from the MBP domain, thus

directing the MBP domain (spiral in Figure 2) to the cytoplasmic side of the inner membrane. Note, this is where the N-terminus of the mature Tet protein is believed to exist in its native state. The "cytoplasmic-MBP fusion" to Tet, named CMT10, is constructed from the expression vector pIH821 (New England Biolabs) which uses the *malE* translation initiation signals to direct expression and the ampicillin resistance gene for selection. Previous studies in this laboratory determined the minimum inhibitory concentration of tetracycline for cells containing the CMT10 construction to be 40 μg/ml. This value for resistance to tetracycline suggests that the MBP domain does not interfere with the metal-tetracycline/H^+ antiporter activity of the Tet domain. The CMT10 MBP-Tet fusion is chosen for insertion analysis for two reasons: (*i*) The fusion protein and its proteolytic digestion products can easily be detected by western blotting with anti-MBP antiserum, allowing structure analysis by proteolysis, and (*ii*) the disruption of tetracycline resistance in mutant transporters may indicate that a structural change in the topology of the mature CMT10 protein had occurred.

II. RESEARCH GOALS AND EXPERIMENTAL DESIGN

This research project is designed to further examine the role of charge distribution across transmembrane segments in governing their orientation of insertion in polytopic (multiple membrane-spanning) *E. coli* membrane proteins, *e.g.*, Tet. It was hypothesized that an increase in positive charge on the C-terminal side of Tet segment E (Figure 2) would alter the topogenic signalling information and alter insertion of this transmembrane segment into the membrane.

The positive charge balance is altered by replacing the neutral amino acids, S^{158}, L^{159}, and H^{160} with basic lysine residues in the periplasmic loop between the transmembrane segments E and F. The third periplasmic loop of Tet is selected for mutation to determine if a hierarchy of interactions between the 12 transmembrane segments can override altered segment insertion signals exerted at a single site. The net charge balance of the second cytoplasmic loop between segments D and E, and the third periplasmic loop between segments E and F are about neutral, $\Delta(C - N) = +1$. While the number of positive residues required to alter insertion signals is not known, three basic lysine residue replacements, $\Delta(C - N) = +4$, are chosen to maximize the effect where segment E should prefer the N_{exo}/C_{cyt} orientation.

Gross topological changes resulting from altering the positive charge balance across hydrophobic membrane-spanning domains have been demonstrated for the simple bitopic asialoglycoprotein receptor subunit H1 (Beltzer *et. al.*, 1991), and the oligotopic (double transmembrane-spanning) leader peptidase protein (von Heijne, 1989). In studies of the polytopic proteins SecY (10 segments, Yamane, *et. al.*, 1990) and MalF (8 segments, Boyd and Beckwith, 1989) by the PhoA fusion technique, net positive charge localizations were found to prevent segment insertion in their normal orientation and to invert hydrophobic transmembrane segments. The SecY and MalF studies conducted with the PhoA fusions, however, have not adequately addressed the influence of possible overriding signals exerted on the affected transmembrane segment by the remainder of the native protein on either side of the mutation site.

Two possible outcomes for how the lysine substitutions for neutral amino acids in the third periplasmic loop will affect the insertion of Tet are (1) the three lysine

substitutions will alter segment E's membrane orientation, suggesting charge difference is the key to getting orientation, or (2) the recombinant protein will have the normal (wild-type) orientation due to topology stabilization by regions distal to the mutation site. To address these affects on Tet insertion, the experimental design has taken five parts:

1. Mutagenesis of the third Tet periplasmic loop

The positive charge balance across segment E is shifted by the substitution of 3 lysine residues into the third periplasmic loop between segments E and F (Figure 2). The mutagenesis is directed to a DNA fragment coding for those segments of the *tet* gene which are subcloned into M13.

2. Construction and analysis of the CMT10-K3 expression vector

The double-stranded form of the *tet'* DNA fragment containing the 3-lysine (K3) substitution is removed from the M13 vector and put into CMT10 to make the CMT10-K3 expression vector. The insertion behavior of the K3 protein is defined based on its digestion at proteolysis sites normally found in the second and third cytoplasmic loops of Tet. Since the CMT10 protein is resistant up to 40 μg/ml tetracycline, the structure of CMT10-K3 is compared by testing for a change in tetracycline sensitivity.

3. Construction of CMT10 deletion marker-protein expression vectors

Truncated versions of the cytoplasmic MBP fusion are needed as proteolytic

mapping markers to further define the precise proteolytic cleavage sites of CMT10 and CMT10-K3. The CMTΔE and CMTΔS proteins constructed from Δ*EagI* and Δ*SalI* deletions (Figure 2) have migration rates which match the rates of protease cleavage products derived from cleavage sites in cytoplasmic loops flanking the mutation site.

4. Proteolysis of CMT10 and CMT10-K3 inverted membrane vesicles

Proteolytic digestions of inverted membrane vesicles (IMVs) prepared from wild-type and K3 mutant MBP fusions are performed to determine topological changes resulting from the mutation. Changes in the patterns of MBP-Tet degradation products are presumed to demonstrate changes of topology.

5. Construction and analysis of Tet-AP reporter fusion vectors

Fusion constructions of the alkaline phosphatase (AP) reporter to pBR322 wild-type *tet* and K3 *tet* at the Sal I endonuclease restriction site are made. These constructions, TetS-AP and TetK3S-AP, are compared to see the effects of positive charges on segment orientation in the absence of the downstream Tet region. Orientation of transmembrane segment F (Figure 2) is determined by alkaline phosphatase indicator plates and by alkaline phosphatase assay.

III. MATERIALS

Commercially available kits containing enzymes and competent TG1 cells for performing the oligonucleotide-directed *in vitro* mutagenesis were purchased from

Amersham International plc. The synthetic oligonucleotides (K1, K2, & K3) used for site-directed mutations are prepared by the facilities available at Uniformed Services University of the Health Sciences:

K1: 5'- C GCC ATC AAA TTG CAT G - 3'

K2: 5'- GCC ATC AAA AAG CAT GCA - 3'

K3: 5'- CC ATC AAA AAG AAG GCA CC - 3'

The inserted lysine codons are underlined. Manual DNA sequencing is performed by the chain-termination method using enzymes and substrates from the Sequenase® Version 2.0 sequencing kit, United States Biochemical Corportation. Detection of MBP is based on enzyme-linked immunodetection of anti-MBP antiserum purchased from Promega. The Promega Protoblot® western blot AP system used contains IgG secondary antibody alkaline phosphatase-linked conjugates and alkaline phosphatase substrates BCIP and NBT. DH5α^{TM} cells, used in transformations, are purchased from BRL Laboratories. Reagents used in the lysine-labeling technique are Immunopure® NHS-LC-biotin, and the Immunopure® ABC avidin-biotinylated alkaline phosphatase kit purchased from Pierce Chemical Co.

IV. METHODS

IV.A. Construction of the CMT10-K3 and CMT10-K3ΔS vectors

The M13D1 mutagenesis vector and the CMT10 expression vector (Figure 3) were previously constructed in this laboratory and used as starting materials for this project. The *tet* DNA containing the mutagenesis site (asterisk) located within the

third Tet periplasmic loop was present on a 275 bp *BamHI-SalI* restriction fragment on both vectors.

Initial attempts to replace all three or two of the targeted residues at once failed, perhaps from improper annealing of the mutant oligonucleotides to the M13 template. Therefore, only three nucleotides are changed per trial using the mutagenesis system. In summary, the mutagenesis process involves altering one codon on the M13 vector at a time, propagating the resulting M13 mutant for use as a new template, and repeating the process until the K3 mutant is obtained. After obtaining the-double stranded replicative form (RF) of the K3 mutant, the K3 *tet* fragment is isolated for replacement of the existing wild-type fragment in the CMT10 vector.

IV.A.1. Mutagenesis of the M13 Tet D1 vector

The K3 mutant containing three lysines is constructed sequentially starting with a *tet* mutant having only one lysine replacement, K1. The K1 single-stranded oligonucleotide 17-mer (containing 17 nucleotides) is made to match the wild-type *tet* sequence except for the mutation replacing the serine[158] codon, 5'-TCC-3', with a lysine codon, 5'-AAA-3' (marked with an asterisk, Figure 3). The bacteriophage M13 Tet D1 vector containing the *HindIII* to *SalI tet'* DNA fragment is used as the single-stranded template for mutagenesis. The phosphorothioate oligonucleotide-directed mutagenesis strategy involved in the Amersham system is described briefly as follows:

The K1 oligonucleotide is annealed to the wild-type *tet'* sequence on the M13

Tet D1 template to form a short heteroduplex that serves as a primer for synthesis of the second strand by DNA "Klenow" polymerase. The primer is extended in the presence of the thionucleotide dCTPαS, rendering the mutant strand non-cleavable with *NciI* restriction enzyme. The mutant strand of the heteroduplex is circularized by ligation with T4 DNA ligase. Double-stranded heteroduplex forms are separated from unconverted single-strands by filtration through nitrocellulose and then nicked by *NciI* digestion in the wild-type strand flanking the mutation site. A segment of the wild-type strand corresponding to the mutation site is removed from the heteroduplex by degradation with exonuclease III. Repolymerization of the digested strand with DNA polymerase I and sealing with T4 DNA ligase creates the homoduplex closed circular DNA containing the mutation in both strands. The recombinant M13 replicative form (RF) DNA molecules are then transformed into *E. coli* hosts bearing the F-plasmid (*e.g.* TG1) which is necessary for infection and propagation of M13.

IV.A.2. Propagation of the M13-K1 mutant

The recombinant RF M13 Tet D1 phage DNA is transformed into the host strain TG1 by incubating 300 μl competent TG1 cells (genotype: K12, Δ[*lac-pro*],*supE, thi, hsdD* 5/*F'traD*36, *proA*$^+$*B*$^+$, *laqI*^{q}, *lacZ*ΔM15), with ~1 ng RF M13 mutant DNA on ice for 40 minutes, followed by heat-shock treatment at 42° for 45 seconds, and then incubation on ice for 5 minutes. Transformed cells are plated out in top agar containing a lawn of freshly prepared JM109 cells (genotype: *recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* λ$^-$, Δ*(lac-proAB),* [F', *traD36, proAB, lacI*^{q}ZΔM15], Yanisch-Perron and Messing, 1985) onto previously warmed 2XTY agar

plates (Miller, 1972) and incubated 15 hours at 37°C.

Plaque cores obtained from pasteur pipette stabs of the agar are incubated in 5 ml LB medium containing 100 μl of overnight-grown JM109 host cells. Cells are incubated at 37 °C for 6 hours and collected by centrifugation at 3000xg for 10 minutes. The supernatant contains phage particles with single-stranded M13 DNA while the pelleted cells contain the double-stranded M13 plasmid.

Phage particles are precipitated for 1 hour from the culture by adjusting the supernatant to 4% polyethylene glycol (PEG, 8000 MW) and 0.5M NaCl at 4°C. Bacteriophage particles are recovered by centrifugation at 7,650xg for 12 minutes at 4°C, washed in fresh PEG/NaCl and resuspended in TE buffer (sterilized 10mM Tris-HCl, 1mM EDTA, pH 8.0). Phage DNA is purified by sequentially extracting the pellets with TE saturated phenol, twice with chloroform, and twice with diethyl ether. DNA is ethanol precipitated in the presence of 3M sodium acetate, washed with 70% ethanol, dried and resuspended in 20μl TE. The replacement of serine^{158} with lysine on the *tet* gene insert is confirmed by dideoxy-nucleotide ^{35}S-dATP sequencing of the single-stranded DNA from several plaque clones (Figure 4).

IV.A.3. Generation of the M13-K2 and M13-K3 from the M13-K1 mutant

Single-stranded M13-K1 mutant phage DNA is used as the template for construction of the K2 mutant. The synthetic oligonucleotide 5'-C GCC ATC AAA AAG CAT G-3' encoding two lysines (underlined) at positions 158 and 159 of the Tet protein is used to direct the mutagenesis reaction. The Amersham *in vitro* system is used to generate the homoduplex mutant K2 molecule as described above

for the K1 phage. The presence of two lysine codons at positions 158 and 159 of the *tet'* sequence is confirmed by chain-terminator dideoxy-nucleotide sequencing of the single-stranded plaque clones (Figure 4, K2). The single-stranded K2 phage is likewise scaled up and purified, as described, for use as a template in the generation of the K3 phage. A "K3" oligonucleotide, 5'- CC ATC AAA AAG AAG GCA CC - 3', encoding lysines at positions 158, 159 and 160, is used in combination with the M13-K2 phage template to create the M13-K3 mutant (Figure 4, K3).

IV.A.4. Isolation and purification of restriction fragments

The double-stranded M13 Tet D1-K3 plasmid is obtained from the JM109 host by the alkaline lysis, cesium chloride gradient method described in Maniatis (1982). Fragments from *BamHI* and *SalI* restriction enzyme digestions of the CMT10 and M13 Tet D1-K3 double stranded vectors are separated by electrophoresis at a 7.5 V/cm potential in 0.6% agarose gels containing 0.5 μg/ml ethidium bromide. DNA bands are sliced from the agarose, minced finely and combined with an equal volume of Tris-HCl,(pH 8.0)-equilibrated phenol in microcentrifuge tubes. Extraction of DNA by freeze-thaw and centrifugation at 12,000xg for 15 minutes is repeated by another freeze-thaw extraction of the agarose-phenol centrifugate with a 1/4 volume of water. The aqueous supernatants are collected and further purified by phenol-chloroform extraction (Maniatis, 1982). A 786 bp *SalI-SalI* and a 6,507 bp *SalI-BamHI* fragment are obtained from the *BamHI/SalI* digested CMT10 vector. A third fragment containing the wild-type *BamHI-SalI* 275 bp *tet'* sequence containing the site for mutation is discarded. The 275 bp *BamHI-SalI* replacement fragment

carrying the K3 mutant form of *tet'* (Figure 3, asterisk) is obtained from the M13-K3 replicative form DNA.

IV.A.5. Ligation of CMT10-K3

The 275 bp *BamHI-SalI* M13-K3 fragment, the 786 bp *SalI-SalI* and 6,507 *SalI-BamHI* CMT10 fragments are combined in a 10:3:1 molar ratio respectively. 0.8 μg total DNA fragments is suspended in ligation buffer containing 50 mM Tris-HCl, (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, and 0.5 mg/ml bovine serum albumin. Ligation is performed by incubating the mixture with 2.5 units of T4 DNA ligase for 16 hours at 16 °C. Upon completion, the ligated mixture is chilled on ice awaiting transformation into host cells.

IV.A.6. Transformation of the ligated CMT10-K3

The ligation mixture is diluted 5-fold in 10mM Tris-HCl (pH 7.5) and 1 mM Na_2EDTA. 1 μl of the dilution (containing 1 - 10 ng DNA) is added to 100 μl chilled, DH5α^{TM} competent cells (BRL Gibco research products) with gentle mixing. Incubation of the mixture on ice for 30 minutes is followed by heat-shock at 42°C for 45 seconds. The reaction is placed on ice for 2 minutes, warmed to room temperature by addition of 0.9 ml S.O.C. medium (Maniatis, 1982), then incubated at 37°C for 1 hour while rotating at 40 rpm. The cells are diluted with S.O.C. medium and plated in variable aliquot volumes on LB plates containing 100 μg/ml ampicillin (LB amp^{100}), then incubated overnight at 37°C.

IV.A.7. Screening for the ligated CMT10 plasmid

Fifteen isolated, ampicillin-resistant colonies from the three-way ligation are selected and grown overnight in LB amp^{100}. Each overnight culture is diluted 1:50 in 5 ml LB amp^{100} and grown at 37°C to a mid-log phase (0.2 - 0.3 A_{600}). MBP-Tet protein synthesis is induced by the addition of isopropyl *β-D*-thiogalactopyranoside (IPTG) to 1 mM followed by growth at 37°C for 20 minutes. Cells are chilled on ice and harvested by centrifugation at 1000xg for 20 minutes at 4°C. Pellets are resuspended in sample buffer (Fairbanks, *et.al.* 1971) containing 2-mercaptoethanol to a calculated volume of 4.0 A_{600}. Samples are boiled 5 minutes, chilled on ice, and loaded into a 10% acrylamide bis-acrylamide SDS gel for electrophoresis (Laemmli, 1970). Western blots are made by electrophoretic transfer of the separated proteins from the SDS polyacrylamide gel to a nitrocellulose membrane over 3 hours at 300 milliamps in buffer containing 2.5 mM Tris-HCl (pH 8.4), 19.2 mM glycine, 20% methanol.

Detection of the CMT10 MBP-protein fusions is performed by the Promega ProtoBlot® method. The nitrocellulose filter is blocked in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) containing 3% nonfat dried milk for 30 minutes at room temperature. The blocking solution is replaced with TBST containing 1% bovine serum albumin (BSA) and anti-MBP primary antiserum (New England Biolabs) for 30 minutes at room temperature, washed three times with TBST, and incubated with goat anti-rabbit IgG-alkaline phosphatase (Promega Corporation) in 1% BSA, TBST for 30 minutes. The nitrocellulose is again washed three times and exposed to the color-indicating alkaline phosphatase solution

consisting of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, and the substrates nitro blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). MBP-related proteins turn purple within 1-15 minutes. The reaction is stopped by rinsing with water after sufficient color development to visualize the CMT10K3 clones (Figure 5).

Plasmids from colonies that express MBP-Tet fusions are obtained by alkaline lysis extraction and purified by cesium chloride gradient centrifugation (Maniatis, 1982). The double-stranded plasmids are denatured with 0.2 M NaOH, 0.2 mM EDTA (30 minutes at 37°C) and neutralized by 3 M sodium acetate, pH 5. After precipitation with ethanol at -70°C, and washing the pellet with 70% ethanol and drying, the denatured DNA is redissolved in reaction buffer and annealed with primer for sequencing. The dideoxy nucleotide chain-termination method of sequencing is used to confirm the construction of the CMT10-K3 and CMTΔS-K3 vectors. The CMTΔS-K3 vector is derived from a two-fragment ligation of the vector and the 275 bp insert. The CMT10-K3 and CMTΔS-K3 vectors are thus obtained from a single ligation mixture (Figure 5).

IV.B. Construction of the CMTΔE expression vector

The CMTΔE protein standard is constructed to aid in topology mapping of CMT10-K3. Prior to this research project, this laboratory created an EagI site near the junction of the second cytoplasmic loop and transmembrane segment E (Figure 2) by changing the wild-type *tet* sequence in M13D1 from 5'-GGG CTC-3' to 5'-GGC GCC-3'. The double-stranded *BamHI-SalI* fragment bearing the EagI site is first

removed from the M13 vector, then ligated to the 6,507 bp CMT10 *SalI-BamHI* fragment in a 3:1 molar ratio respectively. The resulting CMTΔS vector containing the *EagI* site is transformed into *E. coli* DH5α™ cells. Plasmid DNA is isolated from ampicillin-resistant colonies by the alkaline lysis - rapid, small-scale method (Maniatis, 1982). Purified plasmids are digested with *EagI* and subjected to electrophoresis in 0.6% agarose, 0.5 μg/ml ethidium bromide to confirm the presence of the *tet'-EagI* insertion.

The construction of CMTΔE is completed by introducing a TAG stop codon near the *EagI* site (Figure 2). To construct this MBP-TetΔE truncation, the purified CMTΔS vector is digested with *EagI*, then filled-in by polymerization with deoxy-nucleotide triphosphates using the large (Klenow) fragment of DNA polymerase I. A commercially available, double-stranded, nonsense *SpeI* linker 5'-CT AGA CTA GTC TAG-3'(New England Biolabs) is phosphorylated using ATP and polynucleotide T4 kinase (Maniatis, 1982). The phosphorylated linker is subsequently blunt-end ligated, using T4 DNA ligase, to the Klenow-filled *EagI* site at a 100-fold molar excess of linker. Excess *SpeI* linkers are removed from the circularized CMTΔE vector by digestion with *SpeI* enzyme. Re-ligation of the remaining *SpeI* site is accomplished with T4 DNA ligase.

After transformation of the CMTΔE ligation mixture into DH5α™ competent cells and growing on LB amp^{100} plates, isolated colonies are screened, as described for the CMT10-K3 screening, by western blot analysis using anti-MBP antiserum. Plasmid is isolated from a colony whose MBP-protein band migrated at the correct MW in a 10% SDS-polyacrylamide gel. The construction of the CMTΔE plasmid is

confirmed by analyzing *SpeI*-digested DNA in a 0.6% agarose, ethidium bromide gel.

IV.C. Construction of pBR322 TetK3S-AP

A *phoA* fusion is made at the *SalI* site of pBR322 *tet* after switching the K3 mutation into pBR322 (Figure 6). The activity of this fusion is compared with a wild-type pBR322 *tet-phoA* fusion. The pBR322 Tet-K3 mutant is made by ligating the 275 bp *BamHI-SalI tet'*-K3 fragment to an agarose-purified 4,087 bp *SalI-BamHI* pBR322 fragment. The ligation mixture is transformed into DH5α^{TM} cells and the pBR322-K3 vector is purified by large-scale alkaline-lysis method. DH5α^{TM} cells harboring this *tet*-K3 plasmid are ampicillin resistant at 100 μg/ml and tetracycline sensitive at 12.5 μg/ml.

Plasmid pBR322-K3 is prepared for insertion of the *phoA* sequence by digesting with *SalI* and blunting the ends with Klenow polymerase. The alkaline phosphatase reporter gene fragment, *phoA*, is obtained from the pSWFII vector (Ehrmann, *et. al.*, 1990) by digesting with *SalI*, purifying the digested DNA by phenol/chloroform extraction, and filling the 3'-overhang with Klenow polymerase. The plasmid is then digested with *EcoRV* and the blunt-ended fragments are separated by electrophoresis in a 0.6% agarose gel. The 1,450 bp *phoA* DNA fragment is extracted from the gel by freeze-thaw and phenol/chloroform purification.

The *phoA* insert is ligated into the *SalI*-filled pBR322-K3 vector by incubating the insert and vector at a 5:1 molar ratio in presence of T4 DNA ligase for 20 hours at room temperature. The ligation mixture is transformed into KS272 (genotype:

Δ*phoA*[*PvuII*]) competent cells (Strauch and Beckwith, 1988), freshly prepared by the calcium chloride method (Maniatis, *et. al.*, 1982). Transformed cells are plated on ampicillin-selective media and incubated 16 hours at 37 °C.

Initial screening for the alkaline phosphatase fusion to pBR322 Tet-K3 is performed by colony-lift hybridization with the ^{32}P-labeled synthetic oligonucleotide 5'-GTG CGG CAG TAA TTT CC-3'. The wild-type KS272 host strain is deleted for that portion of *phoA* (the *PvuII* deletion) in the region where the synthetic oligonucleotide probe sequence occurs (Strauch and Beckwith, 1988). Control KS272 strains bearing the *phoA*$^+$ pSWFII plasmid and the *phoA*$^-$ CMTΔE plasmid are used to determine the wash conditions needed for specific probe hybridization in buffer consisting of 6X SSC, 2X Denhardt's, 0.1% SDS, 0.15 mg/ml tRNA (Maniatis, *et. al.*, 1982). Filters are incubated with probe overnight at 49°C and washed with 6X SSC, 0.1% SDS at 54°C. Isolated colonies are picked from plates corresponding to positive hybridizations with the ^{32}P-labeled probe.

Secondary screening for the *phoA* fusion is performed by streaking the selected colony picks on LB amp^{100} plates containing 25 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (XP). One of the colonies obtained from the initial screening was blue, whereas the control TetS-AP fusion gave white colonies. Plasmid is isolated from the blue colony and examined by restriction enzyme digestion with *HindIII*. Fragments of 2,160 bp and 3,740 bp are obtained, indicating proper orientation of the *phoA* insert within the plasmid. Further confirmation that the blue colony is from pBR322 Tet-K3ΔS-AP is made by digestion with *KpnI* restriction enzyme from which the expected 3,740, 1,540, and 620 bp fragments are obtained.

IV.D. Inverted membrane vesicles preparation

The topology of the MBP-Tet fusions is examined in inverted membrane vesicles. Expression strains are grown at 30°C to 0.7 A_{600nm} units in M9 media (Maniatis, *et. al.*, 1982) containing 2 mM $MgSO_4$, 0.2% glucose, 0.1 mM $CaCl_2$, 0.2% casamino acids, 2 μg/ml thiamine and 100 μg/ml ampicillin. Cells are chilled on ice and harvested by centrifugation at 6,000xg at 4°C. Inverted membrane vesicles (IMV's) are prepared by high pressure lysis using a French pressure cell according to Rosen and Tsuchija, 1979. The published procedure is modified by including a 10 μM protease inhibitor concentration of chymostatin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and a minimum concentration of 10 μM EDTA in all resuspension and storage buffers. Harvested cells are passed twice through the French pressure cylinder at 6,000 psi. IMV's are suspended at 1-2 mg/ml in 5 mM Tris HCl, 70 mM KCl, 0.25 M dithiothreitol, 55% glycerol, pH 7.2 and stored at -20°C. Membrane protein concentration is determined by a dye binding assay (BioRad). IMV's prepared by this method were previously determined to be stable against deterioration for two weeks when stored at -20°C.

IV.E. Proteolysis of inverted membrane vesicles

Limited protease digestions are conducted with IMV's containing the CMT10-K3 mutant and CMT10 MBP-Tet fusions to obtain partial degradation products. IMV's are suspended in 50 mM potassium phosphate, pH 7.3, 5 mM $MgSO_4$, and 0.5 mM dithiothreitol at a membrane protein concentration of 200 μg/ml and are

digested at 37°C. Chymotrypsin and trypsin digestions are performed for 45 minutes at a final enzyme concentration of 1-10 μg/ml. The final protease concentrations and incubation times are 5-44 μg/ml at 15 minutes for proteinase K, and 0.6-12 units/ml at 1 hour for endoproteinase lysine-C. Proteases are inactivated after digestion by addition of PMSF (dissolved in ethanol) to a final concentration of 0.5 mM. Proteins are processed on ice by precipitation with 10% trichloroacetic acid (TCA), and then centrifuged. The pellet is washed once with 5% TCA and once with 80% acetone before drying and resuspending in sample buffer (Fairbanks, *et. al.*, 1971) for SDS-PAGE and immunoblotting.

IV.F. Alkaline phosphatase assay

The pTetK3S-AP plasmid is isolated by alkaline lysis preparation and transformed into *phoA*$^-$ *E. coli* strain CC118 (Manoil and Beckwith, 1985) competent cells. Cultures are grown at 37°C in LB amp^{100} medium to 2-5 $\times$ 10^8 cells/ml (0.28-0.7 A_{600}) and processed for the alkaline phosphatase assay as described by Miller (1972). Enzyme activity is indicated by hydrolysis of *p*-nitrophenyl phosphate and is measured at A_{420}. Turbidity is corrected by measuring the optical density at A_{550} and using the calculation:

$$Miller\ Units = 1000 \times \left(\frac{A_{420} - (1.75 \times A_{550})}{t \times v \times A_{600}} \right)$$

The Miller Units reflect the reaction time (t) in minutes, the volume (v) in ml of culture used in the assay, and the cell density (A_{600}) just before the assay. The units are proportional to the increase in *p*-nitrophenol per minute per bacterium.

IV.G. Spheroplast preparation and treatment for proteolysis

The published procedure of Witholt, *et. al.*, (1976) is modified by excluding the lysozyme treatment and proceding directly into proteolysis: Cells are grown at 37°C to stationary phase in LB medium and diluted 50-fold into M9 media, supplemented as described in IMV's preparation. The diluted culture is subsequently grown at 30°C to late log phase (0.8 A_{600}) and chilled on ice. Harvested cells are suspended at a cell density equivalent to 11 A_{600} in cold 200 mM Tris-HCl (pH 8.0) containing 40 μM each of protease inhibitors chymostatin, leupeptin, pepstatin A, and PMSF. An equal volume of 200 mM Tris-HCl, 1 M sucrose (pH 8.0) and a 0.1% volume of 500 mM EDTA (pH 7.6) is added. The resulting suspension contains a 5.5 A_{600} cell density in 200 mM Tris-HCl, 0.5 mM EDTA and 0.5 M sucrose (TES suspension). Cells are made sensitive to protease penetration by mild osmotic shock. This is done by making a 2-fold dilution of the TES suspension in cold water, mixing and immediately transferring 100 μl aliquots of the 2.75 A_{600} suspension into 100 μl of proteolysis buffer. Spheroplast proteolysis buffer is made isotonic at 0.25 M sucrose, and contains 0.05 M potassium phosphate (pH 7.3) and 0.5 mM DTT. Exclusion of Mg^{2+} maintains the cells sensitive to protease penetration. Proteolysis is conducted by adding 1-20 μg/ml trypsin and incubating at 37°C for 45 minutes. Digestion is halted by adding PMSF to 0.5 mM and precipitating with 10% TCA.

IV.H. Spheroplast preparation and lysine labeling

E. coli KS474 cells bearing plasmids are grown at 30°C to stationary phase in

M9 media, supplemented as described in IMV's preparation, and containing 30 μg/ml kanamycin. Cultures are diluted 1/20 in fresh media and grown at 30°C to a density of approximately 4 x 10^8 cells/ml (~0.8 A_{600}). Harvested cells are resuspended at a cell density equivalent to 22 A_{600} in cold 50 mM $NaHCO_3$ (pH 8.3), 0.5 M sucrose, containing 40 μM chymostatin, leupeptin, pepstatin A, and PMSF (protease inhibitors). 100 μl aliquots of this suspension are transferred to cold microcentrifuge tubes and treated with 1 μl of 50 mM EDTA (pH 7.6) each. The EDTA-sensitive cellsare subjected o mild osmotic shock by adding100 μl o cold water containing 10 mg/ml Immunopure® NHS-LC-biotin and mixing gently. The lysine labeling reaction is incubated on ice for 30 minutes, then quenched by adding 100 μl of 2 M Tris-HCL (pH 8.0), vortexing and incubating an additional 5 minutes on ice. Labeled spheroplasts are pelleted at 4000xg, and washed once with cold 10 mM sodium phosphate (pH 7.0), 30 mM NaCl, 1 mM EGTA, 0.25% Tween-20, 10 mM β-mercaptoethanol, and 40 μM protease inhibitors (loading buffer). The cells are resuspended in 1.5 ml fresh loading buffer and frozen on dry ice. Frozen spheroplasts are thawed on ice and sonicated, then centrifuged at 12,000xg for 23 minutes. The supernatant is collected and incubated with a 25 μl packed-volume-equivalent of BSA-blocked amylose resin (3.0 μg/μl MBP binding capacity, New England Biolabs) for 4 hours at 4°C with gentle mixing. Amylose resin bound with MBP-Tet is pelleted and washed with 1 ml of cold loading buffer, pelleted and washed with 1 ml wash buffer: 10 mM sodium phosphate (pH 7.0), 0.5 M NaCl, 1 mM EGTA, 10 mM β-mercaptoethanol, 0.25% Tween-20, and 40 μM protease inhibitors. The pelleted amylose is gently mixed at 4°C for 4 hours with 20 μl of

elution buffer, similar to wash buffer, but containing 0.05% Tween-20 and 10 mM maltose. Eluent containing the MBP-Tet is collected and loaded into sample buffer for SDS-PAGE as described in the proteolysis of IMV's.

Nitrocellulose from the western transfer is blocked in 100 mM Tris-HCL (pH 7.5), 0.9% NaCl, 0.1% Tween-20 (TBST), and 10% nonfat dry milk for 2 hours at 37°C with gentle agitation. The nitrocellulose is drained and washed 3 times with TBST (without milk). The biotinylated lysines are conjugated with alkaline phosphatase reagents for 30 minutes at room temperature using the Immunopure® ABC avidin-biotinylated alkaline phosphatase kit, then washed 3 times with TBST. Alkaline phosphatase staining with NBT and BCIP is done as described in the "Screening for the ligated CMT10" section.

V. RESULTS

V.A. Tetracycline sensitivity of CMT10K3

Strains harboring the CMT10-K3 vector were tested for growth in tetracycline media. Host cells harboring the CMT10-K3 Tet plasmid were resistant to 100 μg/ml ampicillin but failed to grow on LB plates containing 12.5 μg/ml tetracycline. Because CMT10 wild-type grows in media containing up to 40 μg/ml tetracycline, this result suggests that CMT10-K3 may have undergone a change in structure compared to the CMT10 control.

V.B. Proteolysis of CMT10 and CMT10-K3 IMVs

CMT10 and CMT10-K3 inverted membrane vesicles were treated with the proteases chymotrypsin, trypsin, proteinase K, and endoproteinase lysine-C to determine if topological changes in Tet resulted from the K3 mutation. The cytoplasmic loops next to transmembrane segments E and F contain protease cleavage sites for chymotrypsin, proteinase-K, and trypsin. Endoproteinase lysine-C cleaves in the fourth cytoplasmic loop. Thus, several sites in the protein can be screened by proteolysis which aids in topology mapping experiments.

Immunoblots of membrane samples taken before and after proteolysis with the four proteases (Figures 7 & 8) revealed no differences in migration rates between bands arising from the CMT10 and CMT10-K3 IMVs; however, the intensities of the anti-MBP stained bands did differ. The anti-MBP staining intensities of the full-length CMT10-K3 fusion, migrating at 71 kDa, appeared lighter (Figure 7) to almost undetectable (Figure 8) than the CMT10 fusion even in unincubated membranes, although both IMV preparations contained the same amount of total protein. The intensities of intermediate digestion products migrating comparable to the MTΔE and CMTΔS markers at approximately 53 and 60 kDa (Figure 7 & 8) also appeared weaker for the K3 mutant. The limit digestion product migrating at ~ 47 kDa appeared heavier from CMT10-K3 than from CMT10 (Figure 7, lanes 14-18 and Figure 8, lanes 8-12, 16-18). All of the CMT10-K3 IMV immunoblots, including the unincubated K3-IMV (Figure 7, lane 14) appeared to be partially degraded when compared to the CMT10 IMV immunoblots before (lane 5) and after incubation in proteolysis buffer. In summary, the digestion products were similar for the two fusions, but staining intensity, thus, yield of bands was less for CMT10-K3.

V.C. Structure and activity of pBR322 TetS-AP and TetK3S-AP fusions

The structures of the wild-type and K3 region in truncated Tet-PhoA fusions can be determined by examining the alkaline phosphatase activities of the fusions since the activity of the enzyme reflects its cellular location. According to Hoffman and Wright (1985), the function of the AP reporter domain is dependent on its secretion across the cytoplasm. The TetS-AP (wild-type) was hypothesized to have an inactive alkaline phosphatase due to the cytosolic location of the fusion junction. A comparison of AP activity from the wild-type Tet and the K3-mutant AP fusions should indicate whether transmembrane segment F flips in orientation responding to insertion of the K3 mutation (Figure 9). These experiments were performed to complement results from analysis of CMT10-K3, where N-terminal domain interactions with the C-terminal domain may stabilize the orientation of segment F.

The pBR322 TetS-AP wild-type and K3 mutants were compared for alkaline phosphatase activity. The wild-type and K3-AP plasmids were transformed into strain CC118(Δ*phoA20*, Manoil and Beckwith, 1985) and compared with respect to color development in LB-XP plates (Table I). Comparison of alkaline phosphatase activities from XP color-indicator plates suggest that the K3 mutation inverted the orientation of transmembrane segment F (Figure 9). That is, colonies of CC118 cells containing the plasmid pTetK3S-AP were dark blue while those containing pTetS-AP or no plasmid were white. These results were supported by measuring the alkaline phosphatase activities of the strains using a *p*-nitrophenyl phosphate hydrolysis assay (Table II). The K3 mutation conferred a 3.5-fold increase in Miller Unit activity compared to the wild-type fusion.

VI. DISCUSSION

Tet-PhoA and MBP-Tet fusion proteins were modified by substituting lysine residues into the third Tet periplasmic loop between transmembrane segments E and F. Experiments with the truncated PhoA fusion were intended to determine if three lysine replacements could flip the orientation of transmembrane segment F in the absence of the C-terminal Tet domain. Experiments with the MBP-Tet fusion were intended to determine whether the lysine substitutions would alter the topology of Tet in the presence of the C-terminal domain, or would long-range forces stabilize the Tet protein in its native topology.

The first indication that function was affected by the K3 mutation came from the tetracycline sensitivity test on the CMT10 fusion. The decrease in the 40 μg/ml resistance conferred by CMT10 after the introduction of three lysines indicates that the metal-tetracycline/H^+ antiporter activity was disrupted. This may suggest that a change in structure and perhaps topology in the membrane had occurred. However, this result does not directly indicate a topology change since any step in the tetracycline transport process could be affected, *e.g.*, the initial binding of the metal-tetracycline complex to the Tet protein.

Evidence that the K3 mutation altered transmembrane segment orientation in the truncated Tet fusion was the finding of a 3.5-fold enzymatic activity increase for the TetK3S-AP fusion compared to wild-type. This increase directly indicates that the orientation of at least transmembrane segment F and perhaps all or a portion of segment E was inverted (Figure 9). The white to blue conversion on XP indicator media provided sharp contrast between the wild-type sequence and mutant

ΔS-Tet plasmids; however, the 3.5-fold increase in alkaline phosphatase activity from K3 was less than expected. One possible explanation for the small increase in activity from the assay could be provided by Traxler, *et. al.*, (1992). They found from pulse-chase experiments that the only active form of alkaline phosphatase is that which resides in the periplasm; however, the assay reflects the steady-state appearance of a protease-stable alkaline phosphatase moiety. Perhaps the translocation of the PhoA domain of K3 occurs with less efficiency than the normal periplasmic PhoA species, or the K3 fusion is unstable. Nevertheless, the *p*-nitrophenyl phosphate assay demonstrates that the alkaline phosphatase domain is exported to the periplasm, whereas the wild-type TetS-AP enzyme favors the cytosolic location.

A model consistent with the alkaline phosphatase fusions is shown in Figure 9. The C-terminal PhoA fusion to wild-type TetΔS (TetS-AP) having minimal PhoA activity is believed to localize the alkaline phosphatase enzyme in the cytoplasm. The introduction of three basic residues to the Tet periplasmic loop (TetK3S-AP) would serve to anchor those positive charges in the cytoplasm, thus inverting the entire hydrophobic segment F and exporting alkaline phosphatase to the periplasm where it becomes enzymatically active. Segment E may bend into the bilayer, although the actual conformation and membrane penetration of this hydrophobic segment is not known. Similar results from alkaline phophatase fusions to multiple membrane spanning proteins such as SecY (Yamane *et.al.*, 1990), MalF (Boyd and Beckwith, 1989), and leader peptidase (Laws and Dalbey, 1989) support this model where the most positive end of the transmembrane segment remains in the cytosol.

Based on the treatment of IMV's containing the CMT10 and CMT10-K3 fusion proteins with all four proteases, the evidence was inconclusive about the nature of any topology change that may have resulted from the 3-lysine mutation; however, the fusion stability data did suggest that some structural changes occurred. Proteolysis products from the chymotrypsin and trypsin digestions (Figure 7) migrated at the same respective rates for both fusions. The full length CMT10 fusion migrated at 71 kDa, based on the mobilities of molecular weight markers and standards. The major chymotrypsin product (CT_1) and trypsin digested bands (T_1 and T_2, Figure 2) appeared at 60 and 54 kDa. Proteinase K products appeared at 59 kDa and endoproteinase lysine-C (KC) appeared at 53 kDa (Figure 8). The darkest KC band appeared in CMT10 IMV's at 64 kDa but was conspicuously absent for CMT10-K3, as were the 59 kDa bands. The expected endoproteinase lysine-C band resulting from an inversion of segment E (Figure 2) did not appear in the CMT10-K3 lanes (16-18) migrating at 59 kDa, just below the MTΔN marker. This could be either that the new site, while inverted, is inaccessible, or that no inversion had occurred. Thus, there was no direct evidence for the inversion of segment F in CMT10-K3.

The CMT10-K3 IMV's appeared less stable in proteolysis buffer than the CMT10 IMV's. In comparing the unincubated IMV's, lanes 5 and 14 (Figure 7), there are differences in the amounts of the full length 71 kDa proteins and what appears to be partially degraded MBP-proteins. Proteinase K and endoproteinase lysine-C digestions revealed, as in the chymotrypsin and trypsin digests, a lack of different bands derived from CMT10-K3, and greater abundance of lower molecular weight degradation products. One explanation for these differences could be that

endogenous enzymes degrade the CMT10-K3 faster than the CMT10 fusion. The CMT10-K3 fusions appeared to have already contained similar degradation products as those obtained from the proteolysis of CMT10. One exception was the lack of a trypsin (T_1) doublet at approximately 60 kDa in the CMT10-K3 fusion corresponding to multiple cleavages in the large cytosolic loop. Taken collectively, the disappearance of the full length CMT10-K3 fusion at 71 kDa and the appearance of a proportionately higher amount of degradation products in the CMT10-K3 IMV's upon and even prior to incubation indicate that the CMT10-K3 fusion is less stable than the CMT10 fusion. A loss of stability may imply some conformational change, although the type of change is not known. If one of the transmembrane domains is not inserted into the membrane and remains on the cytoplasmic surface, the inverted membrane will yield a greater number of accessible regions for proteolytic attack than if the protein were properly inserted. The lack of stability may also indicate a problem in the assembly of the protein.

Since direct evidence for re-orientation of segment F could not be obtained with IMV's, spheroplasts were prepared from fusions CMT10 and CMT10-K3 to identify the topology of the K3 site from outside the membrane. By monitoring β-galactosidase and MBP leakage experiments, the published spheroplast preparation (Witholt, *et. al.*, 1976) was improved to minimize spheroplast cell lysis while providing protease access to the outer plasma membrane. KS474 strains, deficient in membrane proteases defined by the *degP* locus (Strauch, *et. al.*, 1989), were used to improve protein stability. Mock-digests were made by suspending spheroplast KS474 cells containing CMT10 and CMT10-K3 plasmids in isotonic proteolysis buffer

and incubating without added proteolytic enzymes. Anti-MBP western blot analysis made from precipitates of unincubated CMT10-K3 spheroplasts were barely visible (data not shown). Precipitates from incubated mock-digests of CMT10-K3 spheroplasts could not be detected. However, CMT10 spheroplast MBP-precipitates, when suspended at an identical cell density, were clearly visible. Again, the instability of the lysine-mutated MBP-Tet protein was revealed. Due to the instability of CMT10-K3 in spheroplasts, proteolysis mapping experiments could not be performed using this method.

Another attempt to localize the K3 mutation site from outside the plasma membrane was made by a lysine labeling procedure using spheroplasts. KS474 cells containing CMT10 and CMT10-K3 were treated with EDTA and osmotic shocked, as described in methods, to permeabilize the outer membranes for reaction with the lysine-specific biotinylated reagent Immunopure® NHS-LC-biotin. This sulfonated derivative of N-hydroxy succinamide is membrane impermeable and reacts with primary amines. Labeled spheroplasts were broken by sonication and adsorbed on an amylose resin to isolate MBP-Tet fusions. The fusion proteins were eluted from amylose resin using maltose, processed by SDS-PAGE, and western blotted. Biotinylated lysines were detected by an avidin alkaline phosphatase complex.

CMT10 and CMT10-K3 spheroplasts were separated into two portions, each, after the western transfer to examine half by anti-MBP immunoblotting and the other half by avidin alkaline phosphatase visualization. Only CMT10, and not CMT10-K3, was seen from anti-MBP immunoblotting (data not shown). The full-length wild-type fusion protein was not seen by lysine-labeling, perhaps because it lacks lysines on the

outer plasma membrane surface (Figure 2). The CMT10-K3 full-length fusion was not detected by either MBP or lysine-labeling. This result suggests that CMT10-K3 is degraded faster than CMT10 in spheroplasts.

VII. CONCLUSION

The results presented in this study are consistent with the conclusion that when a positive charge balance is shifted across a transmembrane segment, a change in the function and structure of Tet results in the *E. coli* membrane. Replacement of three neutral amino acid residues with three lysines in the periplasm will not only disrupt the tetracycline resistance, but will invert at least one hydrophobic transmembrane segment in the truncated PhoA reporter-fusion, and render the full-length MBP fusion-protein unstable.

The instability of the CMT10-K3 suggests that some structural change has occured. Similar proteolysis fragments from CMT10 and CMT10-K3 suggest that if there is a change in structure, it may be a minor bending of hydrophobic segments instead of a major inversion of segments E and F since other regions of the protein were unaffected. Another possibility could be that segments E and F are not inserted and remain in the cytoplasm.

This research supports von Heijne's transmembrane charge difference method of predicting the hydrophobic segment orientation in a truncated form of the polytopic Tet membrane protein. The bacterial cytoplasmic membrane extrudes protons, possibly leading to a cytoplasmic side-negative membrane potential, and a ΔpH, cytoplasmic side-alkaline. These forces may be responsible for stabilizing

positive charges in the cytosol. However, it is uncertain if an inversion has taken place in the full-length Tet resulting from the positive charge shift. The loss of tetracycline resistance and structural instability, as compared to the wild-type, implies that segments E and F of the K3 mutant are altered if not fully inverted. Thus, the hypothesis that signalling information and insertion of one of the transmembrane segments would be affected upon changing the positive charge balance in Tet appears correct for the truncated form and possibly for the full-length protein. The extent of control on insertion behavior arising from sequences distal to the flanking loops of segments E and F remains unknown. Forces from within the cell may inhibit the translocation of positive charges.

The final answer to structural changes may come from using the lysine-labeling technique to label from outside the membrane. If protein stability does not allow detection of the CMT10-K3, perhaps the K1 and K2 mutants may be structurally amenable to study. The K1 and K2 mutants can also be used to determine the minimum value of charge difference required for altering the stability and structure of Tet. Additionally, similar PhoA fusions to the ΔS-truncated forms of K1 and K2 could be used to determine the value of minimum positive charge required to flip segment F.

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Table I.

Properties of pTetS-AP and pTetK3S-AP on XP indicator media

Host Strain/Plasmid	LB-XP	LB-XP ampicillin
CC118	white	no growth
CC118/pTetS-AP	white	white
CC118/pTetK3S-AP	dark blue	dark blue

Table II.

PhoA enzymatic activities of pTetS-AP and pTetK3S-AP

Strain Host/Plasmid	Activity Miller Units
CC118	0.06
CC118/pTetS-AP	0.40
CC118/pTetK3S-AP	1.47

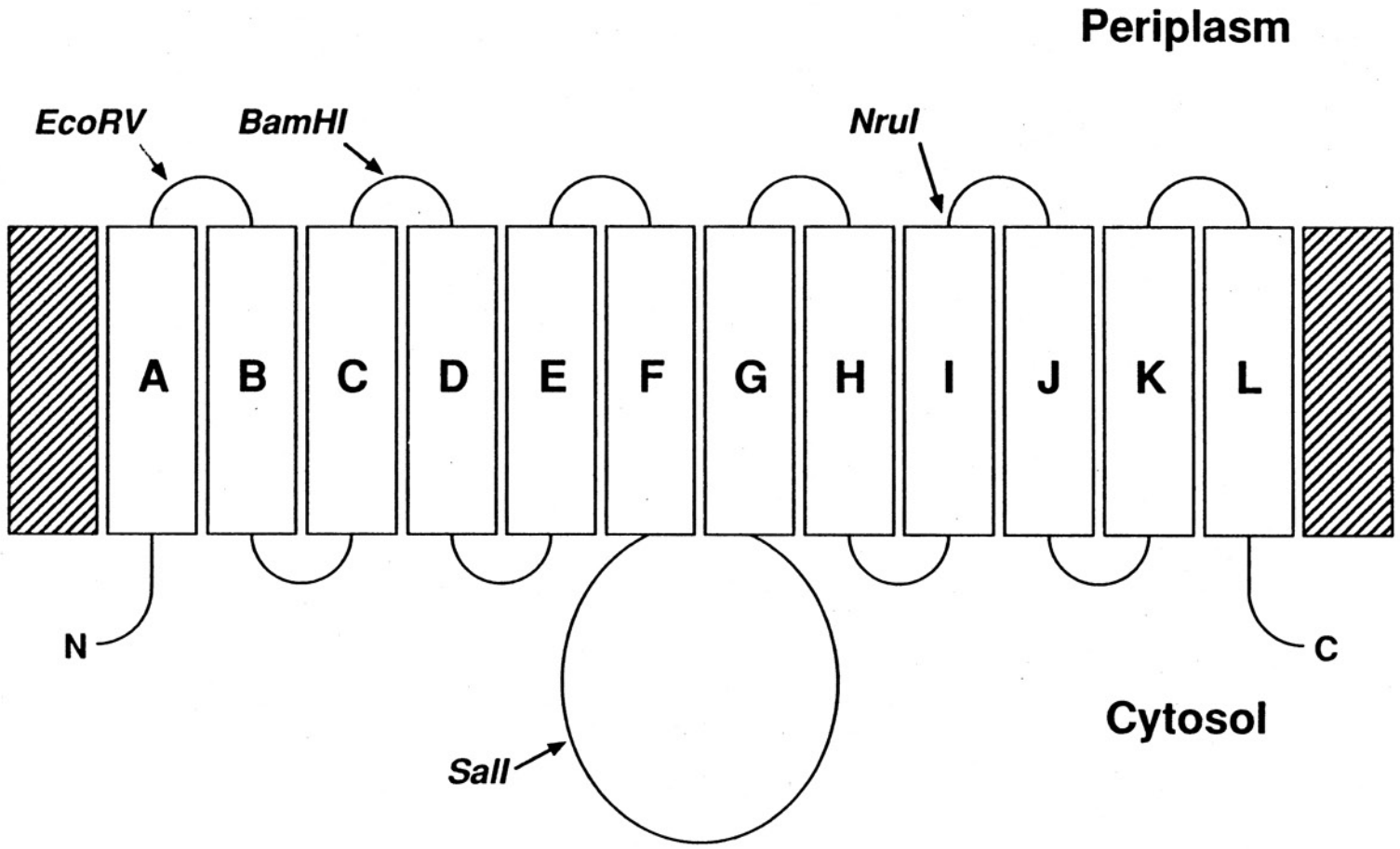

Figure 1 Schematic diagram of the 12 transmembrane-spanning segment tetracycline resistance protein with restriction site locations.

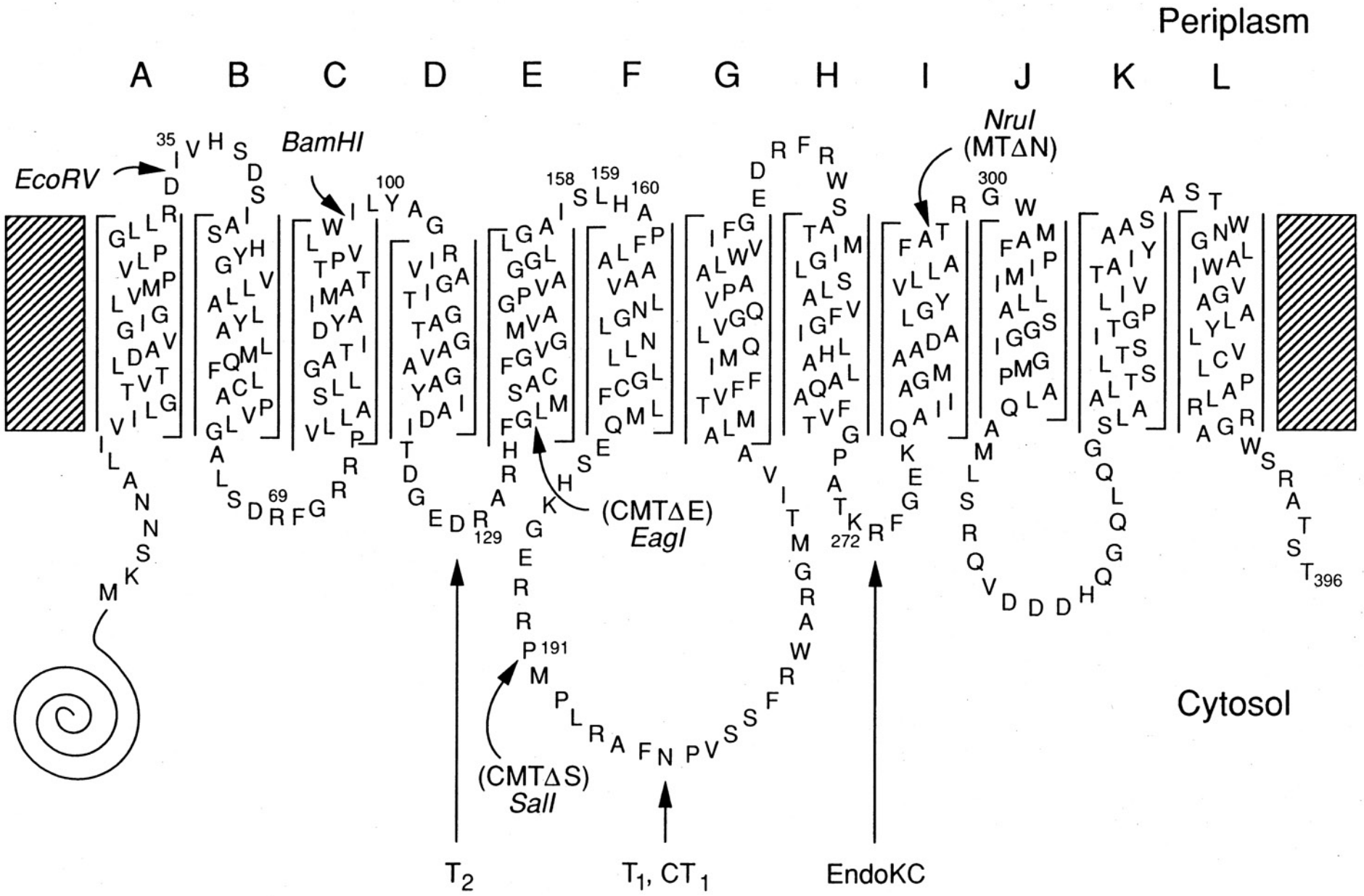

Figure 2 Amino acid sequence of Tet. Includes restriction sites (italics), protein truncation sites (parenthesis), and MBP domain (spiral).

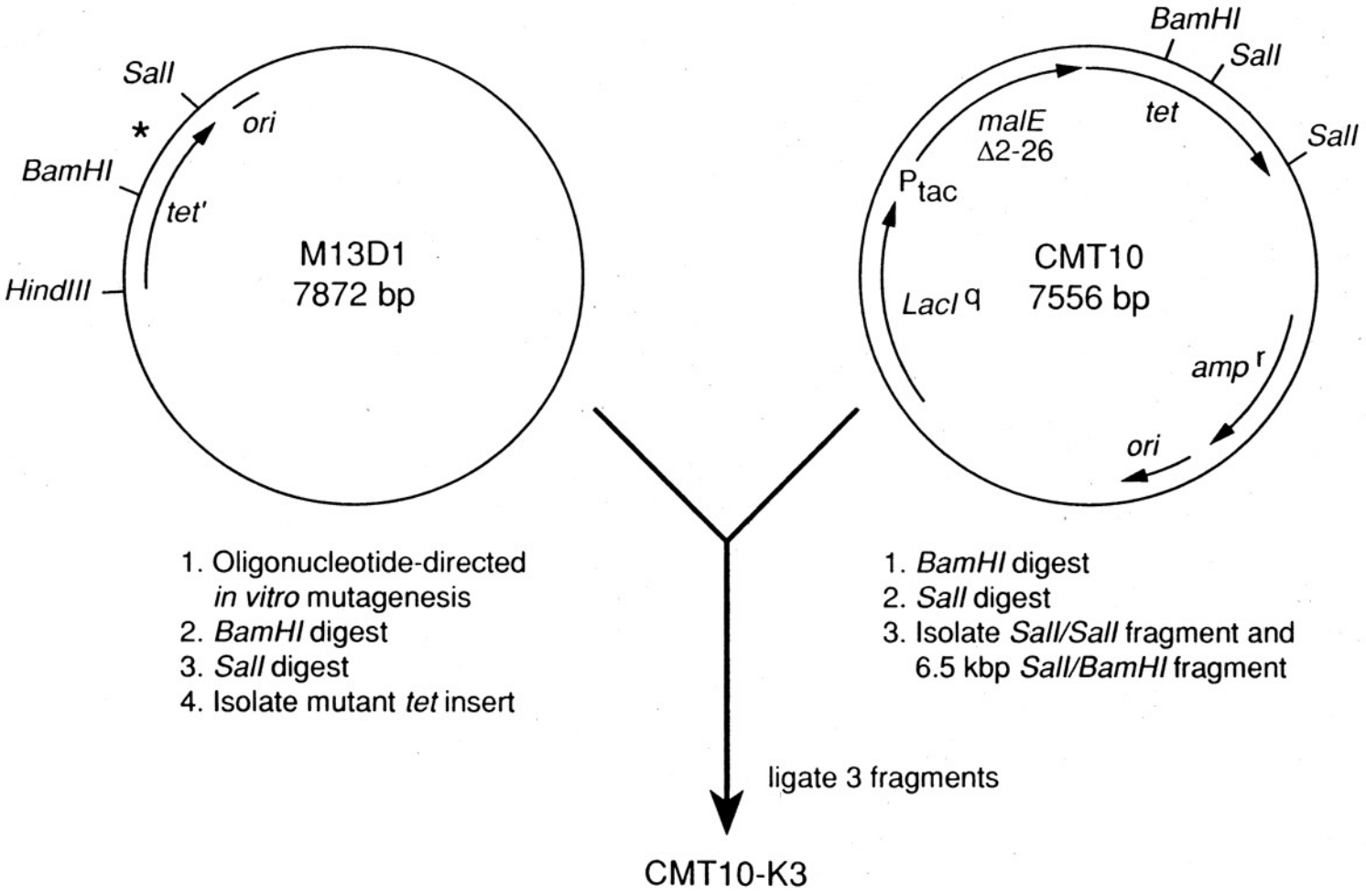

Figure 3 Plasmid construction scheme for CMT10-K3. M13D1 is the single-stranded M13 mutagenesis vector and CMT10 is the fusion protein expression vector.

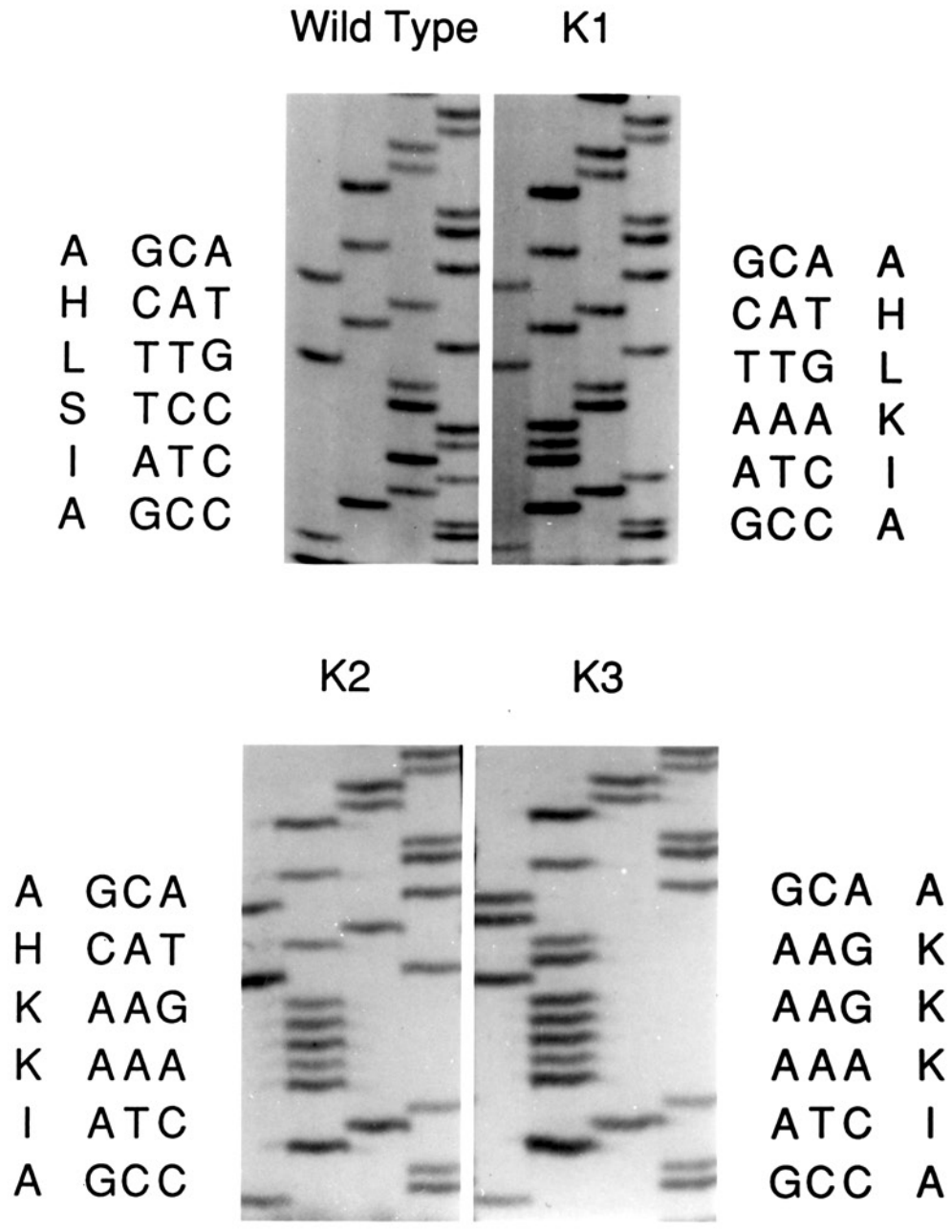

Figure 4 Autoradiograms showing the DNA sequence and how the K3 mutant was sequentially constructed starting with the wild-type sequence and progressing through K1 and K2. Nucleotide codons and their amino acid residues are shown at the sides.

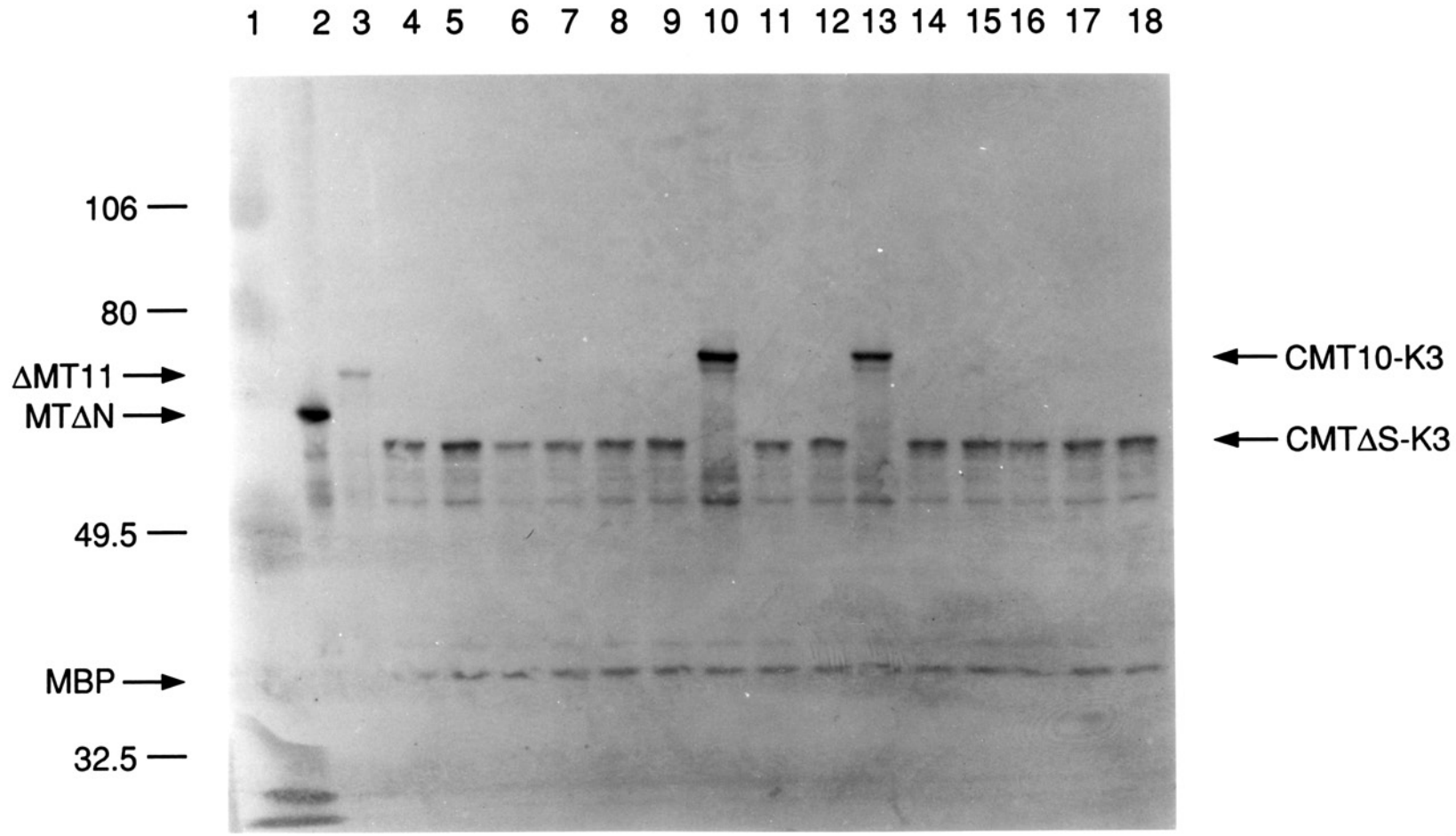

Figure 5. Initial screening for the 3-fragment ligation of the CMT10-K3 plasmid by α-MBP western immunoblot analysis. Lanes 10 and 13 correlated in molecular weight (73 kDa) with the completed ligation of all three DNA fragments of CMT10-K3. Lanes 4-9, 11, 12, and 14-18 appeared to migrate with a molecular weight size (61 kDa) that correlates with the ligation of only two fragments, the 6,507 bp *SalI-BamHI* fragment, and the 275 bp *BamHI-SalI* fragment. The 61 kDa-migrating bands were deduced as the CMTΔS-K3 plasmid products obtained in the absence of the *SalI-SalI* 786 bp fragment.

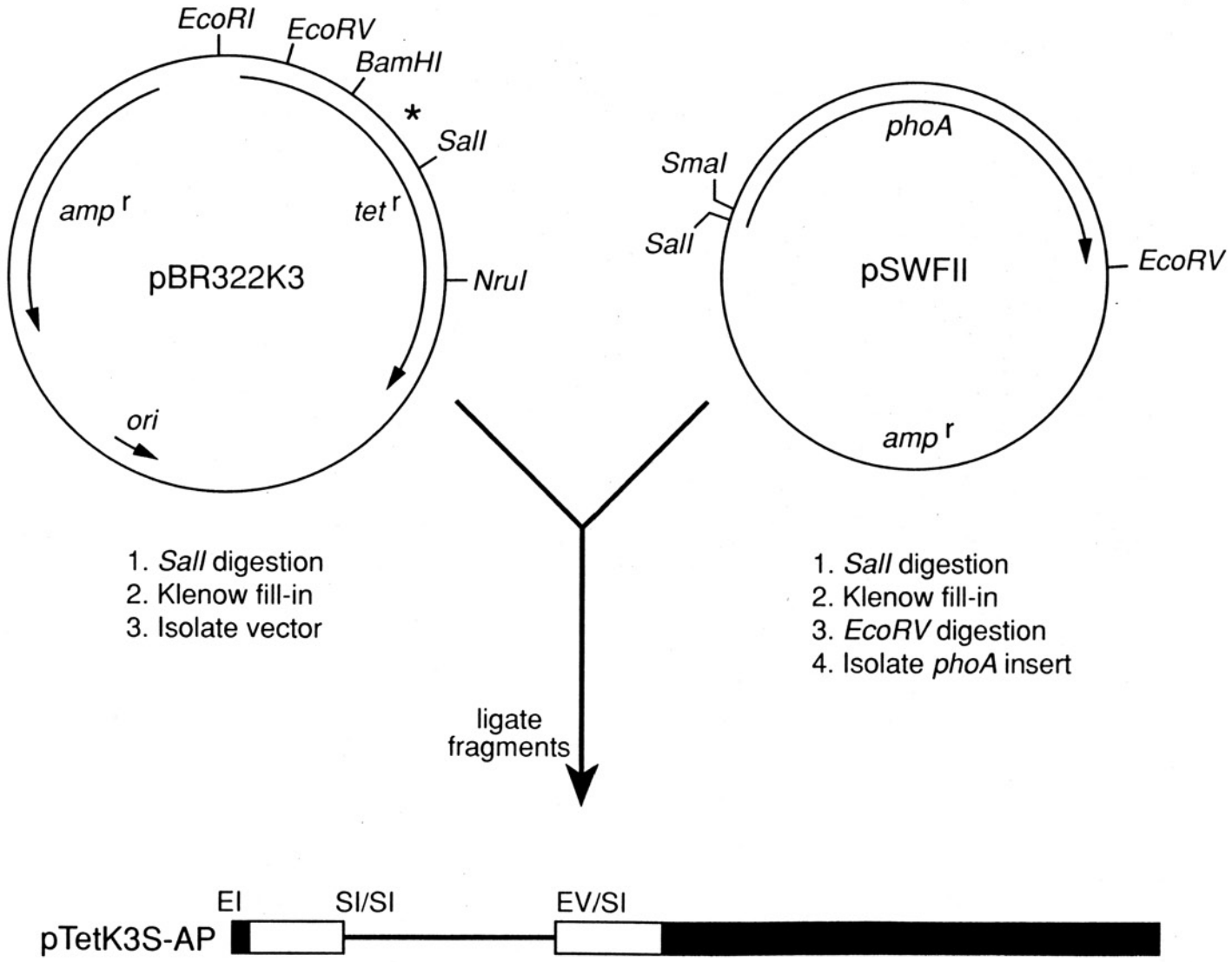

Figure 6 Plasmid construction scheme for TetK3S-AP. The alkaline phosphatase (*phoA*) fragment is removed from pSWFII and inserted (thin line) at the pBR322K3 *SalI* site inside the *tet* region (unshaded bar area).

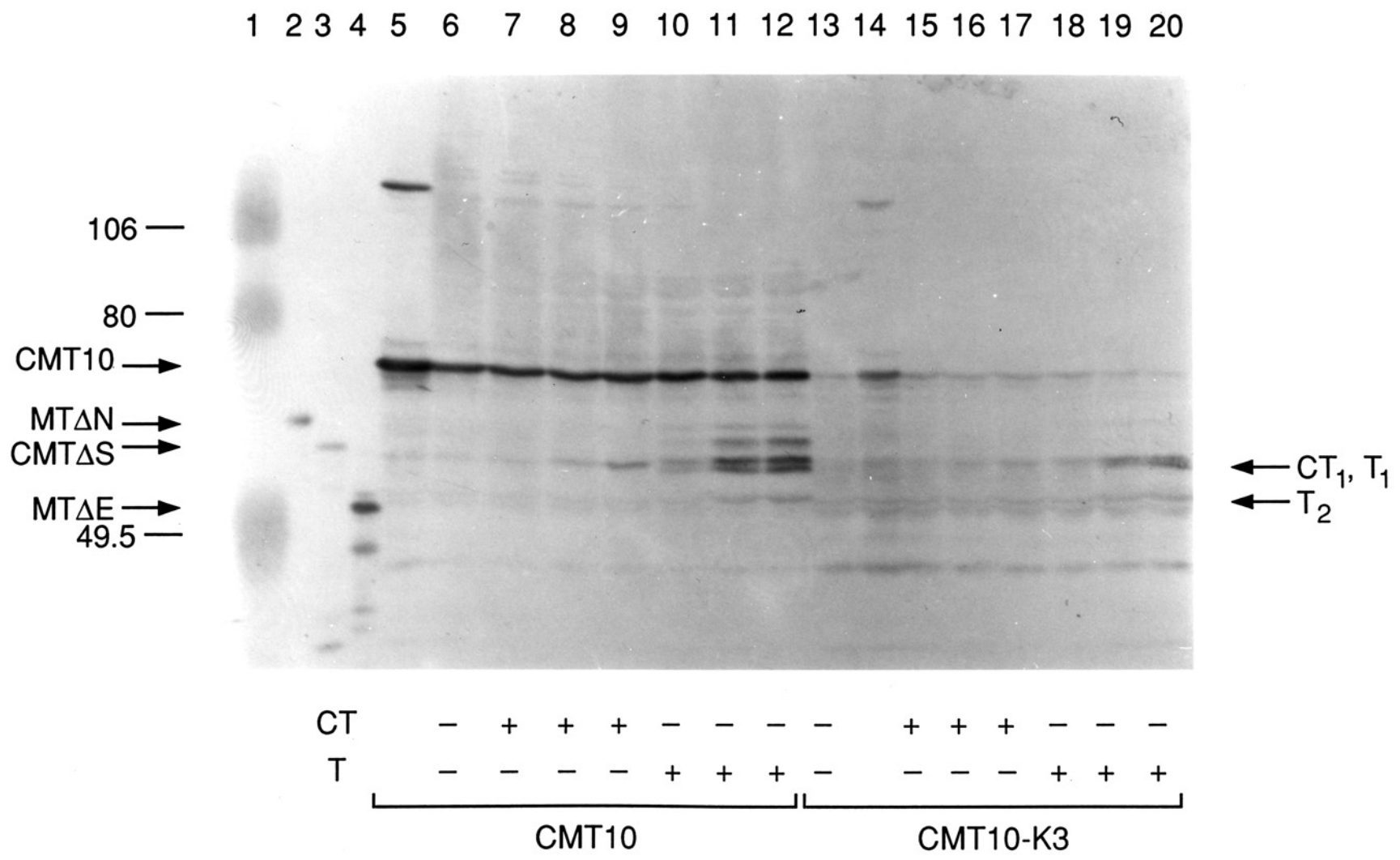

Figure 7 Chymotrypsin (CT) and trypsin (T) proteolysis of IMV's. Enzymes were added (indicated as +) increasing in concentration from left to right. Protein content of the IMV's was assayed to include the same IMV quantity for both fusions. IMV's in lanes 5 and 14 remained in their original suspension buffer on ice for the duration of the proteolysis reaction, and were processed in the same manner as other samples.

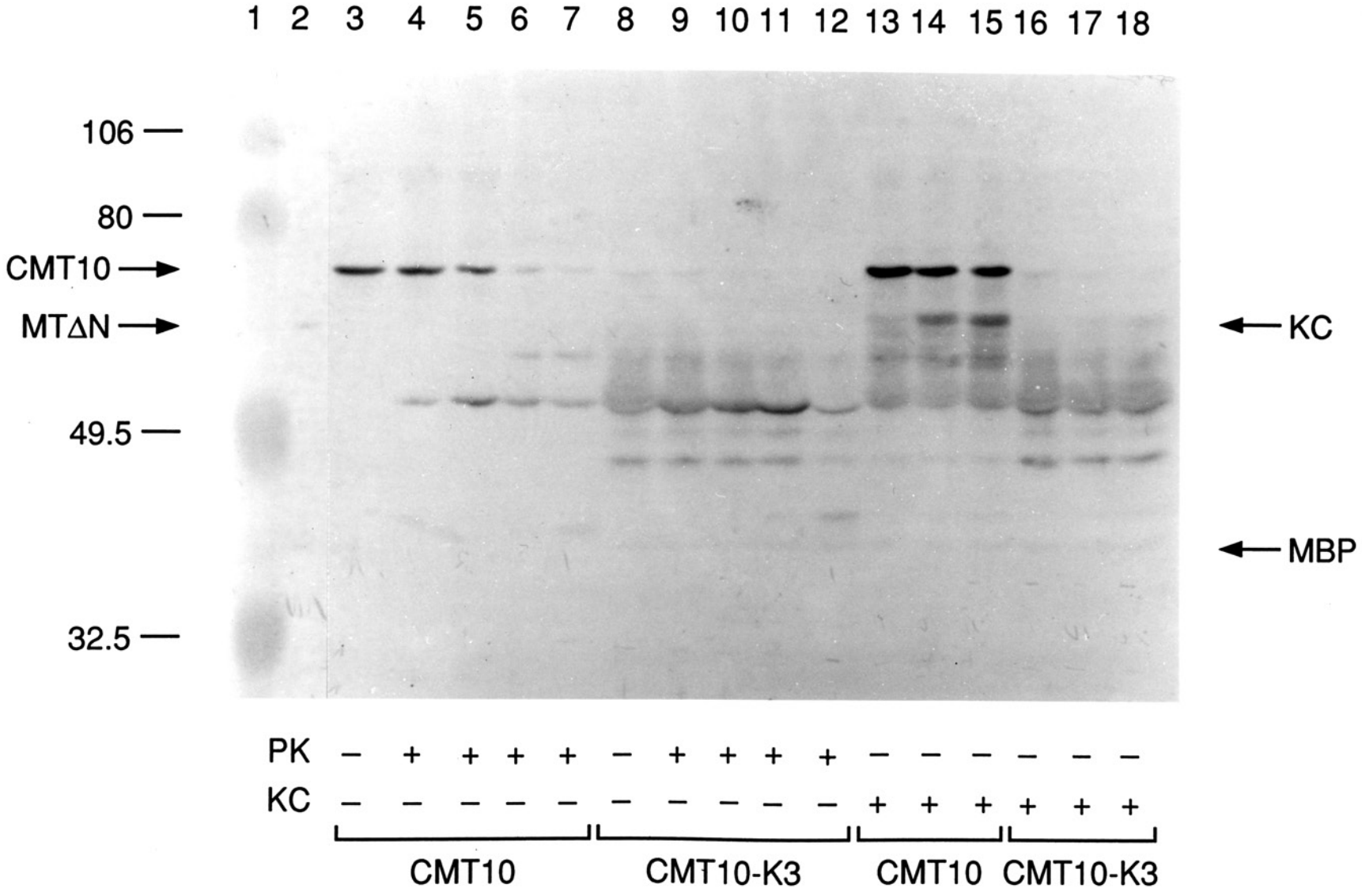

Figure 8 Proteinase K (PK) and endoproteinase lysine-C (KC) digestions of IMV's.

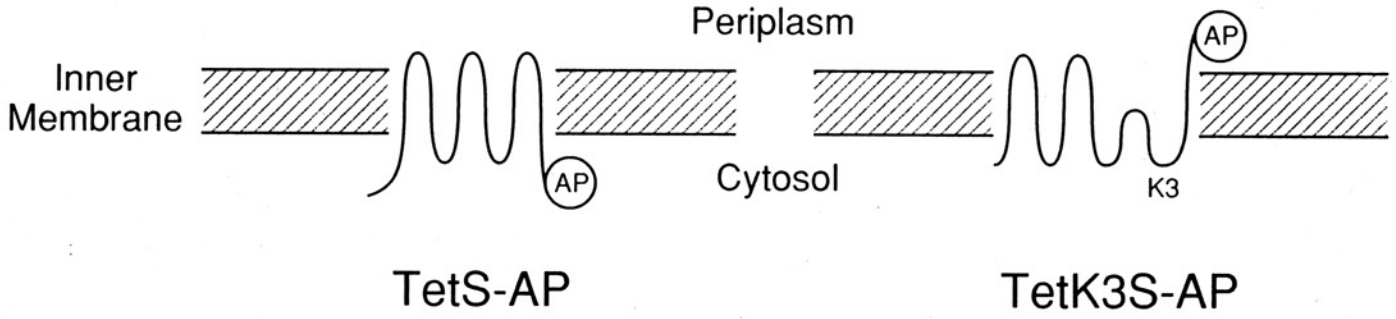

Figure 9 Topology models of TetS-AP and TetK3S-AP. AP represents alkaline phosphatase fused to the truncated pBR322 Tet protein at the *tet* DNA *SalI* restriction site. Wild-type orientation of the sixth transmembrane segment in TetS-AP has no alkaline phosphatase activity, indicating a cytosolic AP. Introduction of basic lysine residues into the third periplasmic loop of TetS-AP leads to an active alkaline phosphatase, suggesting a periplasmic AP where the sixth segment is inverted in orientation and the positive charges are anchored in the cytosol.